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RUBISCO ACTIVITY IS ALTERED IN A STARCHLESS MUTANT OF *NICOTIANA SYLVESTRIS* GROWN IN ELEVATED CARBON DIOXIDE

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Dry matter and net photosynthesis of a wild type and a starchless mutant NS 458 of *Nicotiana sylvestris* (Speg. et Comes) were studied after 25 d of CO_2 enrichment. Plants were grown from seed in controlled environment chambers and treatments of either ambient (35 Pa) or twice ambient (70 Pa) CO_2 were initiated when plants were 3–4 weeks old. Photosynthetic rates measured at 35 and 70 Pa CO_2 and at 900 μmole quanta m^{-2} s^{-1} were unaffected ($P > 0.05$) by 25 d of CO_2 enrichment. However, a CO_2-by-genotype interaction was observed indicating that photosynthetic rates of the wild type but not the mutant at 35 Pa CO_2 differed in response to CO_2 enrichment. Photosynthetic enhancement was greater ($P < 0.001$) in the wild type than in the mutant when the measurement CO_2 was doubled. Total biomass and leaf areas of the mutant and wild type also were unaffected by CO_2 enrichment, although specific leaf weight increased 27% and 13% ($P < 0.001$) for the wild type and mutant lines, respectively. Neither chlorophyll nor soluble leaf protein were affected by CO_2 enrichment. Starch, sucrose, glucose and fructose in wild type and mutant leaf samples were also unaffected by CO_2 enrichment. Rubisco protein levels of the wild type and mutant were about 20% lower in elevated compared to ambient CO_2-grown plants. Initial and total Rubisco activities of wild type and mutant leaf samples were not significantly different ($P > 0.05$) between CO_2 environments. However, initial Rubisco activity was more than 30% lower in mutant than than in wild type samples when results from ambient and elevated CO_2-grown plants were combined. Ribulose 1,5-bisphosphate and 3-phosphoglycerate were 280% and 28% greater in the mutant than in the wild type, respectively. These findings suggested that photosynthesis rates of the mutant were limited by Rubisco activity at 35 Pa CO_2 and that end product synthesis rates limited photosynthesis of the mutant at 70 Pa CO_2.

Key words: Photosynthetic acclimation, CO_2 enrichment, leaf metabolites, *Nicotiana sylvestris*, photosynthate partitioning.

INTRODUCTION

During photosynthesis (P_n), rates of CO_2 fixation are coordinated with rates of end product synthesis.[1,2] Starch and sucrose are the principal end products of P_n in most higher plants. If rates of starch and sucrose synthesis are low relative to CO_2 fixation, then rates of P_n will decrease because inorganic phosphate levels in the chloroplast become limiting for ATP synthesis. If rates of starch and sucrose synthesis are in excess then triose phosphate concentrations will be too low to regenerate the sub-

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strate molecule for CO_2 fixation, ribulose 1,5-bisphosphate (Ru1,5bisP). The biochemical mechanisms responsible for inhibiting CO_2 fixation during end product synthesis limited P_n are not well understood, though deactivation of the CO_2 fixing enzyme, ribulose bisphosphate carboxylase/oxygenase (Rubisco), and decreased levels of inorganic phosphate in the chloroplast have been implicated.[3–5]

Rates of starch and sucrose synthesis usually are sufficient to maintain high rates of P_n under typical conditions employed for plant growth. However, an end product synthesis limitation of P_n can be induced when starch or sucrose biosynthesis is restricted.[2,6] Several species of plants with genetic lesions in the starch biosynthetic pathway have been described[7–9] The mutant (MUT) lines have reduced or trace amounts of leaf starch, elevated soluble sugars and a P_n rate comparable to the wild type (WT) at ambient CO_2. Rates of P_n of a starchless tobacco MUT were decreased relative to the WT in response to short-term elevated CO_2 treatment. Oscillations of P_n and chlorophyll a fluorescence also were observed in the MUT but not the WT upon exposure to high light and CO_2.[10] These observations are consistent with an end product synthesis limitation of P_n, although Rubisco activity has not been examined in the MUT.

A second proposed mechanism of feed back inhibited P_n is that excessive starch and sucrose levels repress the expression of certain photosynthetic genes.[2,11] Decreased levels of Rubisco have been observed in long-term elevated CO_2 experiments and this was correlated with changes in leaf mRNA levels encoding the Rubisco small subunit and other genes.[12,6,11] The photosynthetic responses of both WT and starchless tobacco MUT NS 458 to CO_2 enrichment were examined in the current study. We hypothesized that Rubisco activity in the starchless MUT would be down regulated during growth in elevated CO_2 and that the mechanism would involve either an end product synthesis limitation of P_n or decreased Rubisco protein levels.

MATERIALS AND METHODS

Plant materials

Experiments were performed using WT and MUT line NS 458 of *Nicotiana sylvestris* [Speg. et Comes]. The MUT produces a defective plastid isoform of phosphoglucomutase and contains almost no leaf starch.[8] Seeds were obtained from Dr. Neil McHale, Department of Biochemistry and Genetics, Connecticut Agricultural Experiment Station, New Haven, CT, and were germinated in controlled environment chambers as described earlier.[13] After 25 d growth, 8 WT and 8 MUT seedlings were transferred to individual 3 dm^3 plastic pots filled with equal parts Jiffy Mix (Jiffy Products, Batavia, IL) and vermiculite (WR Grace and Co., Cambridge, MA). Plants were grown for an additional 25 d at 27 °C, 450 $\mu mol\ m^{-2}\ s^{-1}$, PPFD, and either ambient (35 Pa) or twice ambient (70 Pa) CO_2 levels as described previously.[14]

Experimental details

Experiments employed 4 WT and 4 MUT plants each per CO_2 treatment. Following the first harvest, the elevated and ambient CO_2 chambers were reversed and the study was repeated. All measurements were performed 50 d after planting and data from both experiments were combined for analysis. Significant differences were compared by a two-way analysis of variance procedure (SuperANOVA, Abacus Concepts, Berkeley, CA) and n=8, except where indicated. Approximately 2 h after the start of the light period, the ambient and elevated CO_2-grown plants were transferred to matching controlled environment chambers with conditions as for plant growth, except that the irradiance was doubled to 900 μmol quanta $m^{-2}\ s^{-1}$. After a 1 h acclimation period, P_n was measured on the sixth true leaf from emergence at 35 and 70 Pa CO_2, as described previously.[15,13] The initiation of this leaf coincided with the onset of the elevated CO_2 treatment.[8] Increasing the PPFD used for measuring P_n facilitated measuring photosynthetic acclimation to CO_2.[14] The plants were then returned to the respective chambers used for plant growth, and after an additional 2 h acclimation period, leaf discs (0.8 cm^2 each] were removed from the lamina of the leaf that was used to measure P_n. In addition, tissue near the leaf tip was sampled with a freeze clamp apparatus to measure photosynthetic intermediates. Shoots were harvested for dry weight and leaf area analysis when sampling was completed.[15] Ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) activity, Rubisco protein, soluble protein and chlorophyll (Chl) were measured as described

Table 1. Significant differences were determined by two-way analysis of variance. Values are the probability of obtaining a greater F, given equal means. Calculations were based on n=16, except an asterisk indicates n=8.

Parameter	CO_2	genotype	CO_2* genotype
Leaf area	0.1119	0.7369	0.7575
Shoot dry weight	0.6237	0.0190	0.8807
SLW	0.0004	0.0001	0.0788
Chl	0.0585	0.1311	0.8746
Protein	0.7018	0.8313	0.9749
Rubisco protein*	0.0159	0.0466	0.9725
P_n at 35 Pa CO_2	0.0729	0.0887	0.0129
P_n at 70 Pa CO_2	0.2130	0.0001	0.0826
Initial activity	0.7122	0.0294	0.6430
Total activity	0.3598	0.1097	0.6791
Starch	0.3598	0.0001	0.5326
Sucrose	0.9460	0.7564	0.9641
Glucose	0.0539	0.0002	0.7220
Fructose	0.9841	0.0025	0.8915
Ru1,5bisP	0.0759	0.0001	0.0048
3-PGA	0.0262	0.6914	0.9257

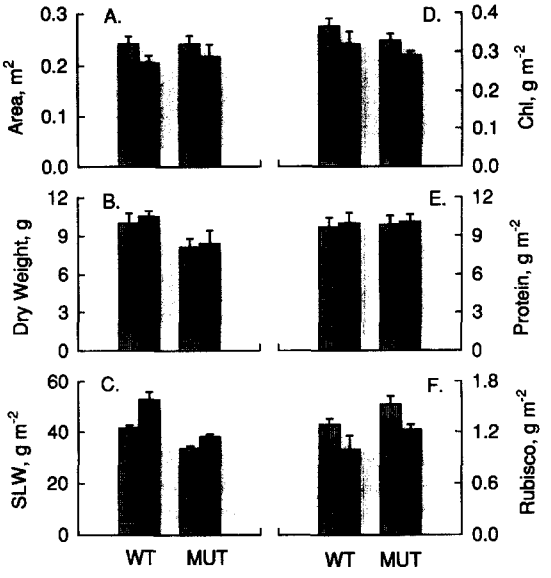

Fig. 1. Effects of CO_2 enrichment on total above ground biomass and on various leaf constituents of WT and starchless MUT NS 458 of *Nicotiana sylvestris*. Differences of leaf area (A), shoot dry weight (B), specific leaf weight (C), Chl (D), total soluble protein (E) and Rubisco protein (F) are shown for ambient (shaded) and elevated (solid) CO_2-grown plants. Vertical bars represent ± SE for n=8.

elsewhere.[14,13] Carbohydrates and the photosynthetic intermediates, Ru1,5bisP and glycerate 3-phosphate (3-PGA), were measured using coupled enzyme assays as described previously.[21,22]

RESULTS

Growth analyses

Neither leaf area nor total shoot dry weight of WT or MUT plants was significantly different ($P>0.05$; Table 1) when plants were grown at 35 and 70 Pa CO_2 (Fig. 1(A) and 1(B)). Averaged over CO_2 treatments, total shoot dry weights of the WT and MUT were 10.3 ± 0.3 and 8.3 ± 0.4 g ($P<0.05$), respectively. In agreement with earlier findings using *N. tabacum*,[16,13] specific leaf weight (SLW) of the MUT and WT was greater ($P<0.001$) at 70 than at 35 Pa CO_2 (Fig. 1(C)). Neither Chl (Fig. 1(D)) nor pheophytin (data not shown) differed ($P>0.05$) in either the WT or MUT with respect to CO_2 treatment. Soluble protein concentrations in WT and MUT plants did not differ ($P>0.05$) between CO_2 treatments, but Rubisco protein decreased about 20% ($P<0.05$) in the WT and MUT when the CO_2 concentration was doubled from 35 to 70 Pa. Rubisco protein concentrations also were greater in WT than in MUT leaf samples ($P<0.05$) when averaged over CO_2 treatments.

P_n and Rubisco actitity

The mean rate of P_n of the ambient CO_2-grown WT was 13.7 ± 0.4 μmol m^{-2} s^{-1}, when measured at 35 Pa CO_2 and at a PPFD of 900 μmol m^{-2} s^{-1} (Table 2). This P_n rate increased 65% in response to a short-term doubling of the CO_2 concentration. The rate of P_n of the elevated CO_2-grown WT was 10.4 ± 1.1 and 19.7 ± 1.3 μmol m^{-2} s^{-1} when measured at 35 and 70 Pa CO_2, respectively. Rates of P_n Of the ambient CO_2-grown MUT were 10.5 ± 0.7 and 16.4 ± 1.1 μmol m^{-2} s^{-1} at 35 and 70 Pa CO_2, respectively. Rates of P_n measured at 35 and 70 Pa CO_2 were unaffected by 21 d of CO_2 enrichment ($P>0.05$), when averaged over both genotypes. However, a significant CO_2 by genotype interaction ($P<0.05$) was detected for P_n rates measured at 35 Pa CO_2. This indicated that P_n rates of the two genotypes responded differently to CO_2

Table 2. Effects of CO_2 enrichment on net photosynthesis and Rubisco activity of wild type (WT) and starchless mutant (MUT) NS458 of Nicotiana sylvestris. *Values are means ± se for* n=8.

Sample	Photosynthetic Rate			Rubisco Activity			Percent Activ.
	35 Pa CO_2	70 Pa CO_2	Δ	Initial	Total	Δ	
		$\mu mol\ m^{-2}\ s^{-1}$					
WT-ambient	13.7±0.4	22.6±0.8	8.9	40.4±6.8	50.1±5.3	9.7	81
WT-elevated	10.4±1.1	19.7±1.3	9.3	36.0±6.3	47.6±7.4	11.6	76
MUT-ambient	10.5±0.7	16.4±1.1	5.9	26.0±3.4	44.0±2.4	18.0	59
MUT-elevated	11.0±0.5	16.9±0.6	5.9	26.5±3.3	37.5±2.6	11.0	71

enrichment. Averaged over CO_2 treatments, P_n of the MUT and WT also differed ($P<0.001$) when rates were measured at 70 Pa CO_2. The ratio of P_n measured at 35 and 70 Pa CO_2, which is an estimate of photosynthetic enhancement, was 1.6 for both ambient CO_2-grown WT and MUT plants, whereas P_n ratios for elevated CO_2-grown plants were 1.9 (WT) and 1.5 (MUT), respectively.

In the current study, Rubisco activity was determined before and after *in vitro* activation with CO_2 and Mg^{2+}. In contrast to similar experiments performed with *N. tabacum*,[14,13] Rubisco was less than fully activated in the WT and MUT leaf samples of the current study. Averaged over CO_2 treatments, initial activity was 78% and 65% of total Rubisco activity in WT and MUT samples ($P<0.05$), respectively. Doubling the elevated CO_2 concentration for 21 d had no affect on either initial or total Rubisco activity, when results for the WT and MUT were combined for analysis. Total Rubisco activities of WT and MUT samples were not significantly different ($P>0.05$), possibly because plant to plant variability was high. However, when averaged over both CO_2 treatments, initial Rubisco activity of MUT samples was 32% less than that of the WT ($P<0.05$).

Leaf metabolites

Effects of CO_2 enrichment on various metabolises in WT and MUT leaf samples are presented in Table 3. None of the principal storage carbohydrates in this study, i.e., starch, sucrose, glucose or fructose, was significantly different ($P>0.05$) when ambient and elevated CO_2 treatments were compared. In agreement with published findings,[8] leaf starch levels were about 10 fold greater in the WT than in the MUT. Present results confirmed that glucose and fructose concentrations were greater ($P<0.05$) in the MUT than in the WT.

Levels of 3-PGA were 25% to 35% greater and whole leaf Ru1,5bisP concentrations were unaffected ($P>0.05$) by doubling the ambient CO_2 partial pressure from 35 to 70 Pa. Levels of Ru1,5bisP were 2–4 fold greater in the MUT than in the WT ($P<0.05$). For ambient CO_2-grown plants, the 3-PGA/Ru1,5bisP ratios were 10.2 and 5.1 for WT and MUT lines, respectively. In comparison, the 3-PGA/Ru1,5bisP ratio was 18.1 and 4.2 for elevated CO_2-grown WT and MUT lines, respectively.

DISCUSSION

We have examined feedback control mechanisms that limit P_n by altering Rubisco activity. Our hypothesis, that a down regulation of Rubisco activity would be observed in the starchless MUT grown at ambient and twice ambient CO_2 partial pressures, was supported. Evidence of decreased Rubisco activity was obtained from gas exchange analysis, Rubisco enzyme and protein assays and metabolite measurements using a starchless tobacco MUT.

According to theory,[1,9] three biochemical processes can impose a limitation on the *in vivo* rate of CO_2 fixation. Inhibition of P_n measured at low internal CO_2 concentrations indicates a Rubisco activity limitation. An inhibition of P_n at above ambient CO_2 suggests that the CO_2 fixation rate was limited by rates of Ru1,5bisP regeneration. An end product synthesis limitation occurs when rates of starch and sucrose synthesis are low and insufficient inorganic phosphate (Pi) is available to sup-

Table 3. Effects of CO_2 enrichment on leaf metabolite concentrations in WT and MUT lines of Nicotiana sylvestris. *Values are means ± se for* n=8.

Sample	Starch	Sucrose	Glucose	Fructose	Ru1,5bisP	3-PGA
		mmol m^{-2}			μmol m^{-2}	
WT-ambient	27.2±3.3	8.6±1.6	4.9±0.6	1.6±0.3	21±2	214±32
WT-elevated	31.0±3.2	8.6±0.8	6.0±0.7	1.6±0.2	15±1	271±16
MUT-ambient	2.9±0.1	9.0±1.9	7.6±0.5	2.5±0.3	40±6	202±23
MUT-elevated	3.7±0.3	9.2±1.7	9.1±0.7	2.6±0.3	62±6	263±27

port photophosphorylation. Evidence of an end product synthesis limitation is derived from a lack of stimulation of P_n when the O_2 concentration is decreased or the CO_2 concentration is increased.[4,5]

In the current study, rates of P_n were measured at the reciprocal CO_2 partial pressures used for plant growth. Averaged over genotypes, rates of P_n measured at 35 and at 70 Pa CO_2 were unaffected by either long-term growth CO_2 treatment. However, a CO_2 by genotype interaction was detected for measurements of P_n performed at 35 Pa CO_2. This indicated that P_n rates of the WT and MUT measured at 35 Pa CO_2 responded differently to CO_2 enrichment. We propose that the differing effects of CO_2 enrichment on rates of P_n of the WT and MUT measured at 35 Pa CO_2 arose because of altered Rubisco activity in the starchless MUT. There was considerable evidence suggesting that Rubisco activity was modified in the MUT. First, Rubisco protein concentrations were lower in the MUT than in the WT. Also, Rubisco protein levels in the WT and MUT were decreased in response to CO_2 enrichment. Reduced Rubisco concentrations in response to CO_2 enrichment have previously been observed in rice,[17] tomato[18,19] and *N. tabacum*.[14,13] Second, activation of Rubisco was decreased in the MUT compared to the WT. Rubisco was only partially activated in *N. sylvestris*, whereas Rubisco was fully activated in both elevated and ambient CO_2-grown *N. tabacum* at a similar PPFD.[13] Third, Ru1,5bisP levels were up to 4-fold greater in the MUT than in the WT. The above findings collectively support the suggestion that decreased rates of P_n of the MUT measured at 35 Pa CO_2 relative to the WT were affected by decreased Rubisco activity.

In agreement with published findings using short-term elevated CO_2 treatments,[10] rates of P_n of ambient and elevated CO_2-grown WT and MUT NS 458 differed at 70 Pa CO_2. Hanson[10] concluded that P_n rates of starchless MUT NS 514 measured at 70 Pa CO_2 were inhibited by rates of end product synthesis. Butz and Sharkey[20] reported that Rubisco was deactivated and Ru1,5bisP accumulated in the chloroplast when P_n was limited by rates of end product synthesis. Both deactivation of Rubisco and elevated Ru1,5bisP levels were observed in the ambient and elevated CO_2-grown starchless MUT relative to the WT. However, rates of P_n by the MUT increased 56% when the measurement CO_2 partial pressure was doubled. Therefore, it was unlikely that rates of P_n of the MUT measured at 35 Pa CO_2 were limited by rates of end product synthesis. An accumulation of Ru1,5bisP and deactivation of Rubisco in the starchless MUT may represent a biochemical adjustment to a defective starch pathway. Sharkey and Vanderveer[5] have argued that a buildup of Ru1,5bisP in the chloroplast should sequester inorganic phosphate, a condition that would stimulate starch biosynthesis in WT plants.

Photosynthetic acclamation is a change of P_n in response to long term (days to weeks) growth in elevated CO_2 and depending upon species can be either positive or negative. In prior studies using *N. tabacum*,[16,14,13] plant dry weight, SLW, and leaf starch increased, and photosynthetic capacity and Rubisco activity decreased in elevated compared to ambient CO_2-grown plants. Acclimation of P_n in *N. tabacum* was strongly negative when measured at 35 and 70 Pa CO_2 both at moderate and at low PPFD. In contrast to the above, evidence of photosynthetic acclimation in elevated CO_2 grown *N. sylvestris* was limited. Shoot biomass of WT and MUT lines of *N. sylvestris* did not differ between the ambient and elevated CO_2 treatments. Starch and sucrose con-

centrations of the WT and MUT also were unaffected by the elevated CO_2 treatment. However, SLW was about 20% greater in elevated than in ambient CO_2-grown plants, indicating that SLW was less variable on a per plant basis than was either dry weight or leaf area. The differing acclimation responses of *N. tabacum* and *N. sylvestris* to CO_2 enrichment can not be explained at this time.

In summary, rates of P_n of ambient and elevated CO_2-grown WT and MUT plants differed when measured at 35 Pa CO_2. The observation that Ru1,5bisP was increased, percent Rubisco activation was decreased and Rubisco protein levels were decreased in the MUT relative to the WT was consistent with the suggestion that P_n in the absence of starch synthesis was limited by Rubisco activity. A Rubisco activity limitation and an end product synthesis limitation of P_n in the MUT were detected for CO_2 fixation rates measured at 35 Pa and at 70 Pa CO_2, respectively. Total biomass, shoot growth and carbohydrate accumulation in *N. sylvestris* were virtually unaffected by 25 d exposure to 70 Pa CO_2 air. There were fewer effects of CO_2 enrichment on *N. sylvestris* than in prior studies using *N. tabacum*.[14,13]

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